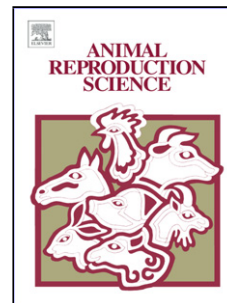


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Title: Interaction between IGF1 and IGFbps in bovine cystic ovarian disease

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1 **Interaction between IGF1 and IGFBPs in bovine cystic ovarian disease**

2

3 **Short title:** IGF1 and IGFBPs in bovine cystic ovaries

4

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21 **ABSTRACT**

22 Cystic ovarian disease (COD) is one of the main factors responsible for reproductive  
23 disorders in cattle. Although the pathogenesis and mechanism of cyst formation are not  
24 fully understood, it has been proposed that the IGF system could play an essential role, as  
25 it is a key intraovarian regulator. The aim of the present study was to determine whether  
26 the altered levels in IGF1 detected in bovines with COD are associated with changes at  
27 mRNA level or with differential modulation by IGFBPs. The mRNA levels of the IGF  
28 components studied were analyzed by real time PCR and in situ hybridization, and IGFBP  
29 expression and activity were assayed by immunohistochemistry and ligand blot  
30 respectively. Results showed a decreased IGF1 mRNA level due to a lower granulosa cell  
31 gene expression in cystic follicles ( $P < 0.05$ ). Results also showed variations in IGFBP  
32 expression in the intraovarian cellular compartment and concentration in follicular fluid,  
33 and suggest that IGFBP3 is a key regulator of intrafollicular IGF1 in animals with COD.

34

35 **Keywords:** cattle, cystic ovarian disease, insulin-like growth factor, ovaries

36

## 37 INTRODUCTION

38 Ovarian follicular cysts in cattle are characterized by large anovular structures that persist  
39 in the ovary for approximately 10 days in the absence of a corpus luteum, with interruption of  
40 the normal estrous cycle (Silvia et al., 2002; Vanholder, 2006). The incidence of this disease has  
41 been estimated to occur in up to 30% of dairy cows, and the number of cases has increased in  
42 recent years (Garverick, 1997; Silvia et al., 2005; Vanholder et al., 2006), probably due to the  
43 selection of high-yielding animals.

44 The pathogenesis and mechanism of cyst formation are not fully understood. One of the  
45 main difficulties in investigating ovarian cysts in bovines is that there are different factors  
46 contributing to cyst formation (Vanholder et al., 2006). However, a hypothalamic  
47 unresponsiveness to an estradiol surge appears to be one of the underlying causes of follicular  
48 cyst formation in dairy cows (Gümen and Wiltbank, 2002). It is believed that an altered  
49 feedback mechanism of estrogens in the hypothalamic-pituitary axis can result in an aberrant  
50 GnRH and LH release and cyst formation, where growth factors play an active role.

51 The insulin-like growth factor (IGF) system is a key intraovarian regulator and the  
52 successful development of a healthy oocyte and appropriate granulosa and theca cell  
53 steroidogenesis in the estrous cycle depend on multiple factors, including the proper function of  
54 this system (Jones and Clemmons, 1995; Spicer, 2004). Disruption of even one component of  
55 this system can lead to abnormal follicular development and function, and compromise the  
56 reproductive capacity by contributing to the development of follicular cysts (Zulu et al., 2002;  
57 Ortega et al., 2008, Rey et al., 2010; Probo et al., 2011).

58 The bioavailability of both IGF1 and IGF2 in the follicle is intimately influenced by the  
59 relative proportions of IGF-binding proteins (IGFBPs) (Firth and Baxter, 2002, Rey et al., 2010;

60 Rodríguez et al., 2011) and by the different affinities between the ligands and individual IGFBPs  
61 (Jones and Clemmons, 1995; Rey et al., 2010). Six IGFBPs (IGFBP1 to 6) have been described to  
62 bind IGF1 and IGF2, prolong their half-lives, and block their action in most situations (Rechler,  
63 1993; Firth and Baxter, 2002). On the other hand, evidence exists for proteolytic enzymes that  
64 degrade and inactivate IGFBPs during follicular development in mammals. These protease-  
65 induced decreases in IGFBPs likely cause increased levels of free IGFs, which stimulate  
66 steroidogenesis and mitogenesis (Spicer, 2004). Therefore, IGFs are regulated at various levels,  
67 with a constantly shifting contribution of endocrine, autocrine and paracrine factors.

68 Given the role of the IGF system in the ovarian function, we hypothesized that an  
69 imbalance in this system may result in ovarian alterations such as cystic ovarian disease (COD).  
70 Therefore, the aim of the present study was to determine whether the altered levels of IGF1  
71 previously detected in cattle with COD (Ortega et al., 2008) are associated with changes at  
72 mRNA level or with a differential modulation by IGFBPs.

73

## 74 **MATERIALS AND METHODS**

### 75 Induced cystic follicles and controls

76 All procedures were carried out according to the Guide for the Care and Use of  
77 Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Science  
78 Societies, 1999) and the protocol was approved by the Ethics and Safety Committee of the  
79 Faculty of Veterinary Sciences (Universidad Nacional del Litoral, Argentina). Ten nulliparous  
80 Argentinean Holstein heifers (18 to 24 months old; 400 to 450 kg body weight; maintained  
81 under standard husbandry conditions) with regular estrous cycles according to prior detection  
82 of estrus, rectal palpation and ultrasonography, were used. Their estrous cycles were

83 synchronized using the Ovsynch protocol as described previously (Ortega et al, 2008; Rodríguez  
84 et al., 2011). The day on which estrous behavior was first detected was confirmed by rectal  
85 examination and ultrasonography, and designated Day 0 of the cycle (Gümen et al., 2003).  
86 Beginning on Day 15 of a synchronized estrous cycle, five heifers received subcutaneous  
87 injections of tetracosactrin hexaacetate (1 mg/ml/animal; Synacthen Depot, Novartis, Basel,  
88 Switzerland), a synthetic polypeptide with ACTH activity, every 12 h for 7 days (Ortega et al.,  
89 2008). Five control animals received saline (1 ml) (Dobson et al., 2000; Ortega et al., 2008).  
90 Ultrasonographic ovarian examinations were performed in all animals, as previously described,  
91 using a real-time, B-mode scanner equipped with a 7.5 MHz, linear-array, transrectal transducer  
92 (Aloka, SSD 500; Wallingford, CT, USA) (Sirois and Fortune, 1988; Rodríguez et al., 2011). Daily  
93 ovarian ultrasonography was performed throughout one complete estrous cycle (21 days in the  
94 control group) and from Day 14 (Day 0 = day of estrus) until Day 48 (treated group). Cysts  
95 detected by ultrasonography were defined as any follicular structure equal to or greater than 20  
96 mm in diameter that was present for 10 days without forming a corpus luteum (CL) (Dobson et  
97 al., 2000; Silvia et al., 2002). The first day of cyst formation was identified retrospectively, 10  
98 days before the daily detection of a follicle equal to or greater than 20 mm in diameter. The  
99 ovaries were removed 10 days later, for the identification of follicular cysts, by flank  
100 laparotomy. Control heifers were ovariectomized, to obtain normal growing follicles  
101 (approximately Day 18), when the dominant follicle reached a diameter greater than 10 mm.  
102 During dissection of the ovaries, the follicular diameter was measured using callipers and  
103 follicular fluid from each follicle was aspirated and stored separately at -20°C. Tertiary (antral)  
104 follicles dissected from normally cycling ovaries were classified as small (< 5 mm), medium-sized  
105 (5–10 mm) or large (> 10 mm) (Parrott and Skinner, 1998). Blood samples were obtained

106 immediately before ovarian excision and centrifuged at 1000 x g for 30 min. The serum  
107 collected was stored at -20 °C until hormonal analysis (parallel studies Ortega et al., 2008; Rey et  
108 al., 2010). Luteinization of cystic follicles was discarded by means of hormonal analysis.  
109 Follicular fluid was aspirated from both the preovulatory follicles of control animals and induced  
110 cystic follicles and then frozen until analysis. The ovaries were fixed in 4 % neutral buffered  
111 formalin.

112

113 Spontaneous cystic ovaries

114 Ovaries with spontaneous cystic follicles were obtained from dairy cows by flank  
115 laparotomy. Eight pluriparous (mean  $3.1 \pm 1.5$  lactations, range 2-7), high-yielding (mean  $25.12$   
116  $\pm 6.23$  kg of milk per day at diagnosis) Argentinean Holstein cows affected by COD were  
117 previously monitored by ultrasonography, and when a follicle  $\geq 20$  mm in diameter persisting for  
118 10 days in the absence of a functional CL was detected, animals were ovariectomized. Cystic  
119 fluids from animals with COD were aspirated using a probe of the real-time, B-mode scanner  
120 equipped with a 7.5 MHz, linear-array, transrectal transducer (Aloka, SSD 500; Wallingford, CT,  
121 USA) before ovarian excision. After excision, samples were collected, refrigerated on ice and  
122 immediately transported to the laboratory. Follicular cysts were evaluated grossly,  
123 microscopically and by hormone analysis. Tissue fractions of ovaries from cystic follicles were  
124 immediately frozen at -80 °C until use in gene expression assays. Additional sections of ovarian  
125 tissue were fixed in 4 % neutral buffered formalin.

126

127 Tissue preparation

128 For light microscopy, the fixed tissues were dehydrated and embedded in paraffin wax.  
129 Sections (4  $\mu\text{m}$  in thickness) were mounted on slides previously treated with 3-  
130 aminopropyltriethoxysilane (Sigma-Aldrich, St. Louis, MO, USA) and assigned for staining with  
131 hematoxylin and eosin for preliminary observations of all ovarian structures (Salvetti et al.,  
132 2004) or for use in immunohistochemistry (IHC) or in situ hybridization (ISH). Follicles were  
133 classified into primary, secondary, tertiary and cystic follicles (Silvia et al., 2002).

134

#### 135 RNA extraction

136 Total RNA was isolated from the follicular wall of different sized tertiary control follicles  
137 and cysts, after treatment with Trizol LS reagent (Invitrogen, Life technology, CA, USA),  
138 according to the manufacturer's instructions but with slight modifications (Rey et al., 2010;  
139 Rodríguez et al., 2011).

140

#### 141 Reverse transcription

142 To avoid putative genomic DNA contamination, RNA samples were treated with DNase  
143 (Invitrogen) according to the manufacturer's instructions. First strand cDNA was synthesized  
144 using a master mix (Moloney Murine Leukemia Virus (MMLV) buffer, dithiothreitol (DTT),  
145 RNAout, MMLV reverse transcriptase, deoxyribonucleotide triphosphate (dNTP) and random  
146 primers (Invitrogen)). The reverse transcription conditions were as described previously  
147 (Rodríguez et al., 2011).

148

#### 149 Real Time PCR



150 An optimized real time PCR protocol was used to analyze the mRNA expression of IGF1,  
151 IGFBP1, IGFBP4, IGFBP5 and IGFBP6 using SYBR Green I (Invitrogen) technology in LightCycler  
152 (Pfaffl et al., 2001). The IGFBP2 and IGFBP3 mRNA had been analyzed in a previous work  
153 (Rodríguez et al., 2011).

154 Transcript levels were measured by relative quantitative real time PCR using a StepOne  
155 Real Time PCR System (Applied Biosystems, Life technology, CA, USA). An optimized protocol  
156 was used: initial denaturation at 95 °C for 10 min, 36 cycles of denaturation at 95 °C for 15 s and  
157 annealing 62 °C (IGF1), 58 °C (IGFBP1), 60 °C (IGFBP4, IGFBP6), 63 °C (IGFBP5), and 52 °C  
158 glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for 20 s, extension at 72 °C for 30 s and  
159 fluorescence reading at 74 °C. All measurements for each sample were performed in duplicate.  
160 The efficiency of PCRs and relative quantities were determined from a six-point standard curve.  
161 Standard curves were constructed from a dilution series of pooled cDNAs (including six dilutions  
162 from 1/5 to 1/160), and PCR efficiency was calculated using the StepOne software v2.2. In  
163 standard curves,  $R^2$ : 0.99, except in the case of GAPDH, where  $R^2$ : 0.98. About 15 ng cDNA was  
164 used for all primers, except for IGFBP-1, where 90 ng was used for each real time PCR reaction,  
165 and was previously quantified by the Qubit method (Invitrogen). For amplification, 4  $\mu$ l of cDNA  
166 was combined with a PerfeCta SYBR Green SuperMix, ROX (Quanta Biosciences, Inc., MD, USA)  
167 in a final volume of 20  $\mu$ l. The primer sequences used are described in Table 1. The GAPDH gene  
168 sequence was included as the housekeeping gene.

169 The mRNA expression levels of genes were recorded as cycle threshold (Ct) values that  
170 corresponded to the number of cycles at which the fluorescence signal can be detected above a  
171 threshold value. The Ct was calculated manually using StepOne v2.2 (Applied Biosystems).  
172 Negative DNA template controls were included in all the assays, and yielded no consistent

173 amplification. Product purity was confirmed by dissociation curves, and random samples were  
174 subjected to agarose gel electrophoresis. Fold change was determined using the  $2^{-\Delta\Delta C_t}$  method  
175 (Livak et al., 2001).

176

177 Nucleotide sequencing

178 The specificities of the PCR products were checked by direct sequencing to ensure  
179 amplification of the correct sequences using the MacroGen Sequencing Service (MacroGen,  
180 Korea). The resulting sequences were verified using the MegAlign Tool in the LASERGENE  
181 software (DNASar, WI, USA).

182

183 In situ hybridization

184 All reagents and materials were RNase-free. Sections were prepared as described above,  
185 but using RNase-free conditions. Single-stranded oligonucleotide probes of 45 bases, end-  
186 labeled with biotin (Invitrogen) were used and, together with those detected previously  
187 (Rodríguez et al., 2011), we assayed the main IGFBPs described in bovines. Probe sequences are  
188 described in Table 2.

189 Probes were diluted to a final concentration of 200 ng/ml in a hybridization solution  
190 (Sigma). The sections were dewaxed, hydrated and endogenous peroxidase activity was  
191 inhibited with 1 %  $H_2O_2$  in methanol. After washing with Tris-buffered saline (TBS) and sterile  
192 water, sections were subjected to microwave pre-treatment in citrate buffer (pH 6.0). After  
193 washing with TBS, the corresponding probe was added to each slide and the sections were  
194 incubated in a humid chamber overnight at 45 °C (Llewellyn et al., 2007). Following incubation,  
195 the slides were washed with TBS and a pre-warmed astringent solution (one wash of saline-

196 sodium citrate (SSC) 2X-50 % formamide-0.05 % Tween for 5 min, followed by two washes of  
197 SSC 2X for 10 min, one wash of SSC 1X for 10 min and one wash of SSC 0.1X for 15 min, all at  
198 45 °C; finally one wash with SSC 0.1X for 5 min at room temperature). The slides were then  
199 rinsed with distilled water and TBS and incubated with extravidin-peroxidase (1:200, Sigma) for  
200 30 min at 25°C. A positive reaction was visualized using 3,3'-diaminobenzidine (DAB, Liquid DAB-  
201 Plus Substrate Kit, Zymed, CA, USA) as the chromogen. Finally, the slides were washed in  
202 distilled water and then counterstained with Mayer's hematoxylin, dehydrated and mounted.  
203 To verify specificity, adjacent negative control sections were subjected to the same procedure,  
204 replacing the probe with hybridization solution or incubating with a sense probe. The same  
205 hybridization temperature, washing and detection conditions were used for the probes used.

206

## 207 Immunohistochemistry

208 Protein expression of IGFBP1, IGFBP4, IGFBP5 and IGFBP6 was detected using polyclonal  
209 antibodies (conditions in Table 3; Novozymes GroPep Ltd, Australia). The extravidin biotin  
210 immunoperoxidase method was carried out as described previously (Ortega et al., 2009; Salvetti  
211 et al., 2009). For antigen visualization, DAB was used as the chromogen. Negative control  
212 sections in which the primary antibody was replaced by a non-immune rabbit serum were  
213 included (Ortega et al., 2009). Some sections were incubated with DAB alone to exclude the  
214 possibility that endogenous peroxidase activity had been unsuccessfully blocked.

215

## 216 Western blotting

217 To test the specificity of the primary antibodies used, bovine tertiary follicles were  
218 homogenized in a radio-immunoprecipitation assay lysis buffer with a protease inhibitor cocktail

219 (Complete Mini Protease Inhibitor Cocktail Tablets, Roche, Mannheim, Germany), as described  
220 previously (Rodríguez et al., 2011). To this end, 40 µg of protein, along with pre-stained  
221 molecular weight markers (GE Healthcare Buckinghamshire, UK) were separated in SDS-PAGE  
222 containing 15 % (w/v) acrylamide-polyacrylamide, according to a procedure previously  
223 described (Ortega et al., 2009). After blotting on nitrocellulose membranes (Hybond ECL  
224 Nitrocellulose Membrane, GE Healthcare), the membranes were blocked with TBS containing  
225 0.05 % v/v Tween20 (TBST, Sigma-Aldrich Corp.) and 5 % w/v non-fat milk, and then incubated  
226 overnight at 4°C with specific primary antibodies. Bound antibody was detected using anti-  
227 rabbit IgG peroxidase antibody (Amersham, Buckinghamshire, UK; 1:500) and ECL plus western  
228 blotting detection reagents (GE Healthcare).

229

230 Western ligand blot

231 Samples of follicular fluid were separated electrophoretically under non-reducing  
232 conditions and transferred onto a nitrocellulose membrane as described previously (Rey et al.,  
233 2010). Membranes were blocked with 5 % w/v bovine serum albumin (Sigma-Aldrich) in TBS and  
234 then incubated overnight at 4°C with 750 ng/ml of biotinylated IGF1 (Novozymes GroPep).  
235 Membranes were washed with TBST, and incubated with a 1:5000 dilution of extravidin-  
236 peroxidase (Sigma-Aldrich) in blocking solution. Before washes with TBST, signal was detected  
237 by chemiluminescence using the ECL-Plus system (GE Healthcare) on hyperfilm-ECL film (GE  
238 Healthcare). Individual binding proteins were identified on the basis of their molecular weights,  
239 as reported previously (Jones and Clemmons, 1995; Nicholas et al., 2002). The molecular weight  
240 reported for IGFBP3 corresponds to a double band of 40 and 44 kDa. IGFBP2 was detected at 34

241 kDa, IGFBP4 has been reported to have a glycosylated and a non-glycosylated form, with  
242 molecular weights of 29 and 24 kDa, respectively, and IGFBP5 was observed at 31 kDa.

243

244 Image analysis

245 Images were analyzed using the Image Pro-Plus 3.0.1 system (Media Cybernetics, Silver  
246 Spring, MA, USA). Images were digitized using a color video camera (Motic 2,000, Motic China  
247 Group, China) mounted on top of a conventional light microscope (Olympus BH-2, Olympus Co.,  
248 Japan) using an objective magnification of  $\times 40$ , as described and validated previously (Ortega et  
249 al., 2009, 2010; Rodríguez et al., 2011). To obtain quantitative data for IHC and ISH labeling of  
250 IGFBPs in the follicular wall, at least three sections were evaluated for each specimen and  
251 antibody or oligonucleotide. The average density (% of positive area) of the IGFBPs antibody or  
252 oligonucleotide reaction was calculated from at least 20 images of each area (granulosa and  
253 theca cells) in each section as a percentage of the total area evaluated through color  
254 segmentation analysis, which extracts objects by locating all objects of a specific color (brown  
255 stain) (Ortega et al., 2009).

256 For the western ligand blot, the exposed films were scanned at 1200 dpi (scanner HP  
257 Officejet J5, 780). The level of each IGFBP was analyzed by densitometry to obtain an integrated  
258 optical density (IOD) value, which was compared between follicular fluid samples from healthy  
259 and cystic ovaries (Rey et al., 2010).

260

261 Statistical analysis

262 A statistical software package (SPSS 11.0 for Windows, SPSS Inc., Chicago, Illinois, USA)  
263 was used to analyze the data. Tests of homogeneity of variance among groups were conducted

264 using Levene's test. The statistical significance of differences between groups of data was  
265 assessed by one-way ANOVA, followed by Duncan's multiple range tests as a multiple  
266 comparison test. A value of  $P < 0.05$  was considered significant. The results are expressed as  
267 mean  $\pm$  SD.

268

## 269 **RESULTS**

### 270 IGF1 and IGFBPs gene expression

271 The identity of the PCR products was confirmed by sequencing (100% homology with  
272 bovine sequences). No differences were detected in GAPDH gene expression between the  
273 different groups evaluated ( $P > 0.05$ ).

274 Levels of mRNA of IGFBP1, IGFBP4, IGFBP5 and IGFBP6 in the complete follicular wall of  
275 control tertiary follicles of different sizes and cystic follicles were similar ( $P > 0.05$ ) (Figure 1).  
276 Moreover, no differences were detected in the levels of IGF1 of tertiary follicles and cystic  
277 structures ( $P > 0.05$ ) (Figure 2A). However, when mRNA expression was analyzed in different  
278 follicular cells by ISH, IGFBP5 was detected mainly in granulosa cells, where the expression  
279 determined in cystic structures was lower than that in growing follicles (Figure 3B and 4).  
280 Growing follicles from both control and cystic ovaries showed a decreasing expression from  
281 primary to tertiary follicles. IGFBP5 mRNA was detected in theca cells of cystic follicles, but a  
282 very low presence or complete absence was detected in theca cells of tertiary follicles.

283 IGFBP4 was detected in granulosa and theca cells, and no differences were observed in  
284 the structures analyzed ( $P > 0.05$ ) (Figure 3). Similarly to that observed in mRNA expression of  
285 IGFBP5, in granulosa cells, IGFBP4 mRNA levels decreased as follicles grew, and the lowest levels  
286 were detected in cystic structures ( $P < 0.05$ ).

287 IGF1 mRNA was detected only in granulosa cells, with similar levels between structures of  
288 the same group (control, spontaneous COD and experimentally induced COD). However,  
289 reduced levels were detected in growing follicles and cysts from ovaries with induced COD  
290 compared with control and spontaneous COD ( $P < 0.05$ ).

291 Structures analyzed in follicles from ovaries with spontaneous COD consistently showed  
292 higher positive labeling than follicles from ovaries with experimentally induced COD, and the  
293 expression pattern was similar in each structure analyzed (Figure 2B, 3).

294

295 Protein expression of IGFBPs

296 Expressions of IGFBP1, IGFBP4, IGFBP5 and IGFBP6 were evaluated in follicles of different  
297 developmental stages. Insulin-like growth factor binding protein 4, IGFBP5 and IGFBP6 were  
298 detected both in granulosa and theca cells, with greater levels in granulosa cells (Figure 6),  
299 whereas IGFBP1 was not observed in either cell type.

300 Both IGFBP4 and IGFBP5 showed the lowest expression levels in granulosa cells of cystic  
301 structures, and the highest expression in primary follicles of control ovaries as well as in ovaries  
302 with COD ( $P < 0.05$ ) (Figure 7). Theca cells of experimentally induced cystic follicles showed  
303 lower expression levels of IGFBP4 than control tertiary follicles ( $P < 0.05$ ) and expression levels  
304 similar to those of spontaneous cystic follicles ( $P > 0.05$ ). Expression levels of IGFBP5 were  
305 similar in the control and induced structures analyzed ( $P > 0.05$ ), and different from those in  
306 spontaneous structures ( $P < 0.05$ ).

307 In contrast, IGFBP6 expression in granulosa cells increased expression from primary to  
308 tertiary follicles, with larger levels in cystic follicles of experimentally induced COD ( $P < 0.05$ )  
309 (Figure 7). Once more, the levels were higher in the structures from spontaneous COD and

310 increased from primary to secondary follicles, and then remained constant through tertiary and  
311 cystic follicles. IGFBP6 was not detected in theca cells.

312

313 Follicular fluid content of IGFBPs

314 The content of IGFBPs in follicular fluid from different sized follicles was identified based  
315 on their molecular weight (Nicholas et al., 2002). The western ligand blot allowed for the  
316 identification of IGFBP2 (34 kDa), a doublet corresponding to IGFBP3 (40-44kDa), IGFBP4 in its  
317 glycosylated (29 kDa) and non-glycosylated forms (24 kDa), and IGFBP5 (31 kDa). Their identities  
318 were confirmed by western blot. The patterns for IGFBP2 and IGFBP3 have been shown in a  
319 previous work (Rodríguez et al., 2011), whereas those for IGFBP4 and IGFBP5 are shown in  
320 Figure 8.

321 IGFBP2 and IGFBP3 were expressed at higher levels in the follicular fluid of the structures  
322 analyzed than the other IGFBPs detected. However, while IGFBP2 levels remained practically  
323 constant in tertiary follicles and cysts ( $P > 0.05$ ), IGFBP3 levels were higher in cysts than in small  
324 tertiary follicles ( $P < 0.05$ ). In contrast, the glycosylated form of IGFBP4 and IGFBP5 showed  
325 higher levels in small tertiary follicles than in cysts and tertiary follicles greater in size ( $P < 0.05$ ).  
326 No differences in the non-glycosylated form of IGFBP4 were detected in the follicles analyzed  
327 (Figure 8).

328

## 329 DISCUSSION

330 The study of the etiopathogenesis of COD in dairy cows has several limitations. The main  
331 difficulties are the fact that bovine COD is a multifactorial disease and there are few  
332 opportunities to follow clinical cases without treatment because farmers need their cows to



333 receive treatment as soon as possible (Ribadu, 2000; Probo et al., 2011). For this last reason,  
334 many studies have been conducted with slaughterhouse material, which lacks the reproductive  
335 history and information of cyst persistence. In the present study, we were able to follow the  
336 reproductive behavior by ultrasonography and rectal palpation of the dairy cows identified as  
337 having COD, and thus to promptly detect persistent follicles and development of cysts.  
338 Moreover, since follicular cysts were recovered from experimental animals at known times after  
339 cyst emergence, analysis of their structure and function was more accurate (Ortega et al., 2008).

340 In the present study, we determined that IGFBP4 and IGFBP5 mRNA levels in granulosa  
341 cells decreased from primary to tertiary follicles in the control group, similarly to that observed  
342 in sheep (Hastie et al., 2004; Hastie and Haresign, 2006). Interestingly, IGFBP4 mRNA was  
343 detected mainly in granulosa cells, but expressed in lower levels in theca cells, contrarily to that  
344 observed by other authors (Armstrong et al., 1998; Roberts and Etcherntkamp, 2003).  
345 Moreover, the reduced levels of IGFBP4 and IGFBP5 detected in granulosa cells of cystic ovaries  
346 were more evident in the experimental model than in spontaneous cysts, probably due to the  
347 differences in the environmental conditions and the time of persistence of the cysts, which  
348 could influence the gene expression of these IGFBPs. Consistently, IGFBP5 protein expression  
349 showed a similar pattern. On the other hand, similar protein levels of IGFBP4 were observed in  
350 the structures from ovaries from spontaneous and induced COD. Differences in mRNA levels  
351 related to protein expression could be due to the hormonal milieu that participates in the  
352 control of the IGFBP system and proteins associated such as IGFBP proteases (Spicer, 2004; Aad  
353 et al., 2006; Sudo et al., 2007). Moreover, it is worth mentioning that levels detected by IHC  
354 correspond to the glycosylated form of IGFBP4, as demonstrated by western blot.

355 In support of the control exerted by steroid hormones on IGFBP production, Spicer and  
356 Chamberlain (2000) observed that estradiol inhibited IGFBP4 production in bovine granulosa  
357 cells in vitro, but did not determine whether this inhibition was due to a change in the  
358 proteolysis of IGFBP4. IGF1, FSH, and/or estradiol induce IGFBP4 proteolysis in cultured human  
359 granulosa cells (Iwashita et al., 1998). In addition, the reduced levels of IGFBP4 and IGFBP5  
360 determined in spontaneous and experimentally induced cystic follicles as compared with those  
361 in primary follicles from both control and COD ovaries are probably associated with the lower  
362 mRNA levels detected, although control by IGFBP proteases cannot be ruled out. It has been  
363 suggested that IGFBP4 production, via changes in intrafollicular mRNA expression, is correlated  
364 with LH regulation and IGFBP4 degradation (Mazerbourg et al., 2001; Hastie and Haresing,  
365 2010). Moreover, both FSH and LH inhibit the expression of IGFBP5 mRNA in ovine follicles,  
366 determining a decrease in the levels of IGFBP5 mRNA (Besnard et al., 1996a; Hastie and  
367 Haresign, 2006a) and protein (Monget et al., 1993; Spicer and Chamberlain, 2002) in follicles as  
368 they increase in diameter (Hastie and Haresing, 2010).

369 Since there is limited information about the patterns of IGFBP1 and IGFBP6 mRNA and  
370 protein expression in the ovary of ruminants, little is known about the elements that regulate  
371 these binding proteins. In the present study, we found no changes in IGFBP1 and IGFBP6 mRNA  
372 levels in the follicular wall of the tertiary and cystic structures analyzed, and detected no protein  
373 expression in any of the follicles analyzed. Because lower levels of IGFBP1 and IGFBP6 mRNA  
374 detected, not expression of those binding proteins mRNA were analyzed by situ hybridization. It  
375 has been reported that IGFBP1 concentrations in serum and follicular fluid are decreased in  
376 women with polycystic ovary syndrome (Thierry van Dessel et al. 1999). In bovines, IGFBP1 is  
377 detected in small quantities in the follicular fluid, probably because it is either poorly expressed

378 or not synthesized within the follicle compartment, entering the basal membrane as a result of  
379 changes (Nicholas et al. 2002). Probably, these changes could explain why we detected mRNA  
380 but not protein. This lack of detection of protein may also be due to the methodology used  
381 (IHC). This may also be true because we failed to detect both IGFBP1 and IGFBP6 in follicular  
382 fluid by ligand blot (Nicholas et al., 2002), but found IGFBP6 in granulosa cells.

383         Although IGFBPs were synthesized intrafollicularly (Armstrong et al., 1998; Voge et al.,  
384 2004; Rodríguez et al., 2011), we analyzed the levels of IGFBPs in follicular fluid, where IGF  
385 availability is effectively regulated. Changes in IGFBP concentrations in bovine follicular fluid  
386 have been documented during follicle growth and development in cattle (de la Sota et al., 1996;  
387 Stewart et al., 1996), sheep (Perks and Wathes, 1996), and pigs (Yuan et al., 1996). Typically,  
388 dominance is associated with decreased follicular fluid concentrations of the binding proteins  
389 with lower molecular weight, i.e. IGFBP4 and IGFBP5 (Austin et al., 2001), in agreement with our  
390 present results, which showed lower protein concentrations in tertiary follicles increasing in  
391 size. The changes in the IGFBP content of follicular fluid could be due to the regulation of IGFBP  
392 production, changes in the activity of specific IGFBP proteases, or changes in the selective  
393 uptake of IGFBPs from the circulation during follicle growth (Armstrong et al., 1998). In fact, we  
394 detected lower gene expression of IGFBP4 and IGFBP5, which was reflected in lower protein  
395 synthesis, while follicles were growing from primary to tertiary and cystic follicles. However, no  
396 changes were detected in mRNA or protein expression levels of control tertiary follicles when  
397 compared with spontaneous cystic follicles. In follicular fluid, a decreasing concentration of  
398 IGFBP5 was detected through tertiary follicles growing in size until cystic structures. Similar  
399 results were obtained with the 29-kDa glycosylated form of IGFBP4, although no changes were

400 detected with the 24-kDa non-glycosylated form. The lower levels detected could result from an  
401 increasing activity or concentration of IGFBP protease.

402 Previously, we detected similar protein expression of IGFBP2 and IGFBP3 in tertiary  
403 control and cystic follicles (Rodríguez et al., 2011). However, the concentration of IGFBP3 in  
404 follicular fluid increased as the control tertiary follicles increased in size, with the highest  
405 concentration found in cystic structures. Therefore, the differences detected in IGFBP3 content  
406 in follicular fluid support the hypothesis of an extrafollicular origin (Echternkamp et al., 1994;  
407 Santiago et al., 2005; Rey et al., 2010), probably responsible for the bioavailability of IGF1.

408 In previous studies, we detected similar serum IGF1 concentrations in controls and  
409 animals with spontaneous or induced cystic ovarian disease (Ortega et al., 2008). Part of the  
410 lack of a direct effect on ovarian activity depends on how much of the circulating IGF1  
411 concentration is truly reflected in the ovaries. IGF1 concentration in follicular fluid has been  
412 found to be lower (Echternkamp et al., 1990; Spicer et al., 1991), equal to (Spicer et al., 1992),  
413 or greater (Spicer et al., 1992; Ortega et al., 2008) than peripheral IGF1 concentration, and  
414 although they are correlated, in some circumstances, such as short-term fasting, liver-derived  
415 IGF1 can be decreased significantly without affecting intrafollicular IGF1 concentration (Spicer et  
416 al., 1992; Velazquez et al., 2008). Furthermore, it has been suggested that IGF1 is involved not  
417 only in the pathogenesis but also in the maintenance of COD in cattle (Zulu et al., 2002; Probo et  
418 al., 2011). In this pathological condition, while IGF1 concentrations are known to be lower  
419 during ovarian cyst formation (Beam and Butler 1997, 1998; Zulu et al. 2002), intrafollicular IGF1  
420 can be even lower than serum concentrations (Ortega et al., 2008). Considering the alterations  
421 in the expression of IGFBP2 and IGFBP3 reported previously (Rodríguez et al., 2011) and the  
422 results of the present study, we suggest that the decreased IGF1 levels could result from

423 alterations in the diffusion rate between the blood and the follicular fluid. These alterations  
424 could be due to differences in the interaction with IGFbps related to gene and protein  
425 expression and/or to induced changes attributable to a decreased IGF1 mRNA in ovaries with  
426 induced COD, causing an imbalance in the IGF system that could modify the circulating levels of  
427 the free fraction of IGF (Rechler and Clemmons, 1998; Hastie and Haresing, 2006; Thomas et al.,  
428 2007).

429

### 430 **CONCLUSION**

431 The results of the present study support that the IGF system has a clear influence in cows  
432 with COD. We suggest that IGF1 is influenced by the action of IGFbps, mainly by IGFbp3 and by  
433 the decreased level of IGF1 mRNA during a COD condition.

434

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445 **LITERATURE CITED**

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**FIGURE LEGENDS**

**Figure 1:** Expression of IGFBP1, IGFBP4, IGFBP5 and IGFBP6 mRNA in small (S), medium (M) and large (L) tertiary follicles and cysts (C). No differences were detected in the quantification of the PCR products of IGFBP1 (**A**), IGFBP4 (**B**), IGFBP5 (**C**) and IGFBP6 (**D**). Values represent the mean  $\pm$  SD.

**Figure 2:** Expression of IGF1 mRNA levels in follicular structures. (**A**) Quantification of IGF1 mRNA in ovarian follicular wall of small (S), medium (M) and large (L) tertiary follicles and cysts (C) by real time PCR. No differences were detected in the PCR products of IGF1 mRNA. (**B**) Analysis of cell population by in situ hybridization showed decreased expression of IGF1 in experimentally induced cysts (black bars) related to control follicles (open bars) without differences in spontaneous cystic ovaries (hatched bars). Values represent the mean  $\pm$  SD. Bars with different superscript letters denote significant differences ( $P < 0.05$ ).

**Figure 3:** Expression of IGFBP4 and IGFBP5 mRNA levels by in situ hybridization. Quantification of IGFBP4 mRNA in granulosa and theca interna cells (**A**) and quantification of IGFBP5 mRNA in granulosa cells (**B**) in developing follicles of control ovaries (open bars), experimentally induced cystic ovaries (black bars) and spontaneous cystic ovaries (hatched bars). Values represent the mean  $\pm$  SD. Bars with different superscript letters denote significant differences ( $P < 0.05$ ). C: control, I: induced cystic ovaries, S: spontaneous cystic ovaries.

**Figure 4:** Representative images of IGFBP4 (**A-C**) and IGFBP5 (**D-F**) mRNA localization by in situ hybridization in bovine follicles. The immunoreactivity was intense in granulosa cells of tertiary control follicles (**A, D**) and weaker in cystic follicles (**B, E**). No staining was observed when the sense probe was pre-incubated with the antisense probe as negative control (**C, F**). In the right column, the immunopositive areas of **A** and **D** were segmented by digital image analysis, showing in black the differential cytoplasmic immunostaining pattern. Bars = 25  $\mu\text{m}$

**Figure 5:** Representative images of IGF1 mRNA localization by in situ hybridization in bovine follicles. The immunoreactivity was intense in granulosa cells of secondary (**A**) and tertiary (**B**) control follicles and weaker in cystic follicles (**C**). No staining was observed when the sense probe was pre-incubated with the antisense probe as negative control (**D**). Bars = 25  $\mu\text{m}$ .

**Figure 6:** Representative images of IGFBP4 (**A-C**), IGFBP5 (**D-F**) and IGFBP6 (**G-I**) protein localization by immunohistochemistry in bovine follicles. The immunoreactivity was intense in granulosa cells and reduced in theca cells in the follicular structures analyzed. Images represent tertiary control follicles (**A, D, G**) and cystic follicles (**B, E, H**). No staining was observed when the primary antibody was replaced with normal rabbit serum (negative control **C, F, I**). Verification of antibody specificity by western blot analysis of an ovarian homogenate demonstrating the specificity of the antibody is shown on the left. In the right column, the immunopositive areas of **A, D** and **G** were segmented by digital

image analysis, showing in black the differential cytoplasmic immunostaining pattern.

Bars = 25  $\mu$ m

**Figure 7:** Immunohistochemical staining of IGFBP4 (**A**), IGFBP5 (**B**) and IGFBP6 (**C**), showing the percentage of immunopositive areas in granulosa and theca interna cells evaluated in primary, secondary, and tertiary follicles of control ovaries (open bars), experimentally induced cystic ovaries (black bars) and spontaneous cystic ovaries (hatched bars). Values represent the mean  $\pm$  SD. Bars with different superscript letters denote significant differences ( $P < 0.05$ ) in separate granulosa and theca cell compartments. The theca interna was not labeled for IGFBP6.

**Figure 8:** Detection of IGFBPs in follicular fluid by western ligand blot of healthy small (S), medium (M) and large (L) tertiary follicles compared with cystic follicles (C). **A:** Quantification of IGFBP subtypes as determined by molecular weight: IGFBP4 non-glycosylated form of 24 kDa (open bars), IGFBP4 glycosylated form of 29 kDa (squared bars), IGFBP5 (horizontally hatched bars) of 31 kDa, IGFBP2 (black bars) of 34 kDa and IGFBP3 (diagonally hatched bars). Values represent the mean  $\pm$  SD. Bars with different superscript letters denote significant differences ( $P < 0.05$ ) between different follicle types. **B:** Representative western ligand blot showing the IGFBPs that bind to IGF1.



Table 1: Forward and reverse primer sequences (5'→3')

Primer	Sequence (5'→3')	Length, bp	Reference
IGF1	For <sup>1</sup> TCG CAT CTC TTC TAT CTG GCC CTG T Rev <sup>2</sup> GCA GTA CAT CTC CAG CCT CCT CAG A	240	Pfaffl et al. (2002)
IGFBP1	For TCA AGA AGT GGA AGG AGC CCT Rev AAT CCA TTC TTG TTG CAG TTT	123	Pfaffl et al. (2002)
IGFBP4	For GCC CTG TGG GGT GTA CAC Rev TGC AGC TCA CTC TGG CAG	342	Plath- Gabler et al. (2001)
IGFBP5	For TGC GAG CTG GTC AAG GAG Rev TCC TCT GCC ATC TCG GAG	257	Plath- Gabler et al. (2001)
IGFBP6	For AGA AAG AGG ATT TGC CTT TGC Rev TCC GGT AGA AGC CCC TAT G	324	Plath- Gabler et al. (2001)
GAPDH	For CAC CCT CAA GAT TGT CAG CA Rev GGT CAT AAG TCC CTC CAC GA	103	Shibaya et al. (2007)

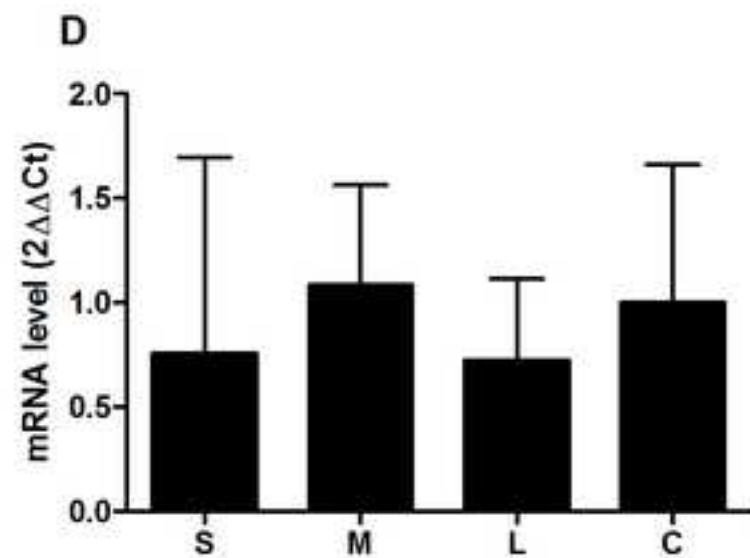
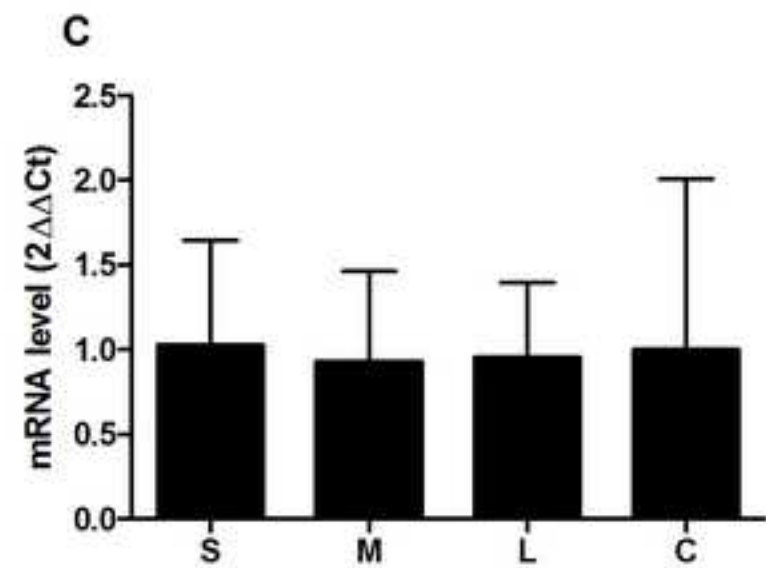
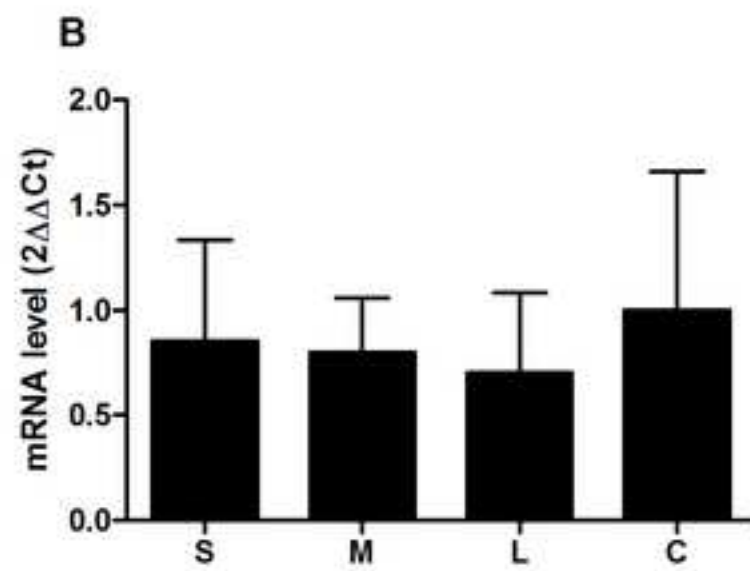
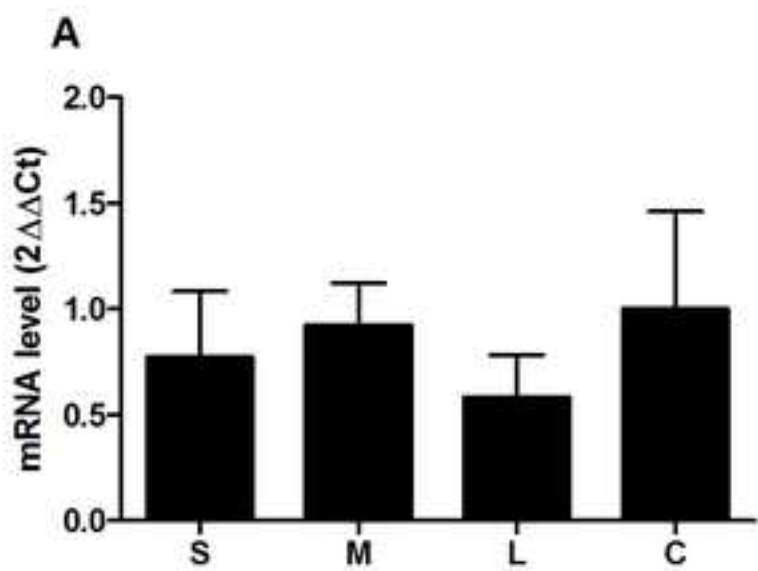
<sup>1</sup> Forward (For)<sup>2</sup> Reverse (Rev)

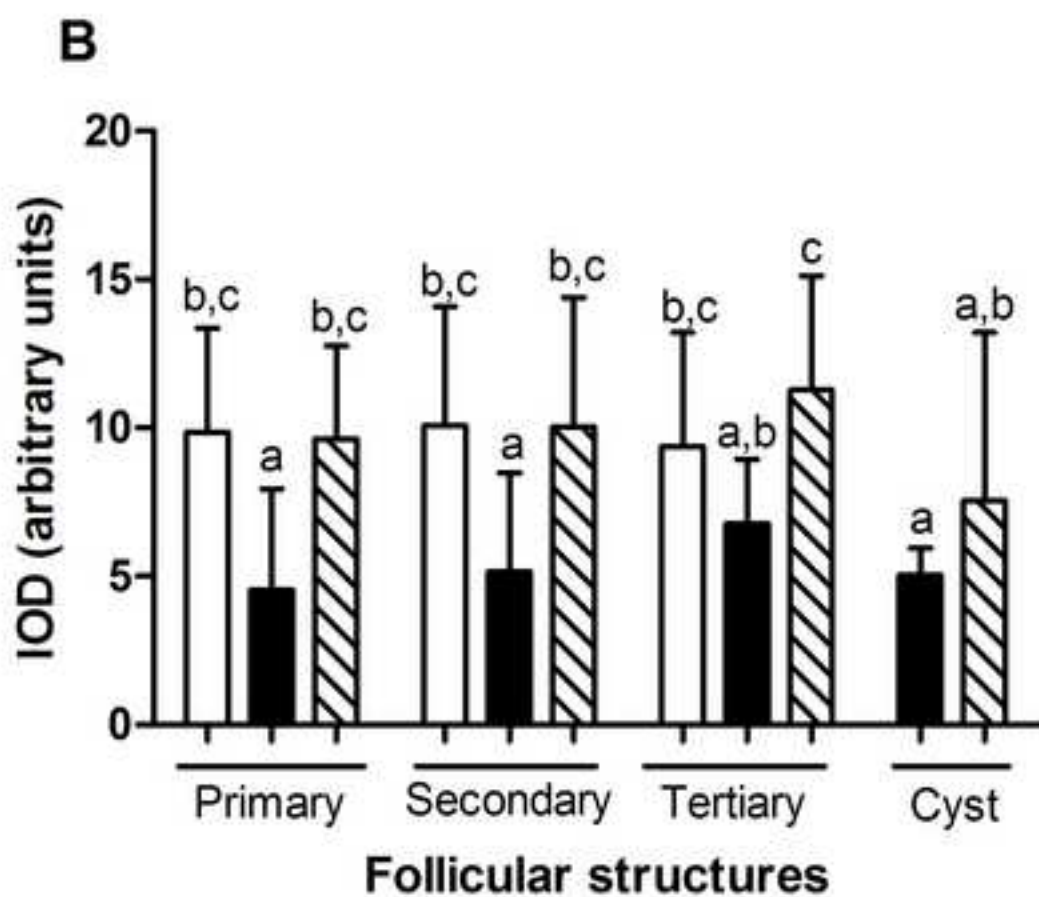
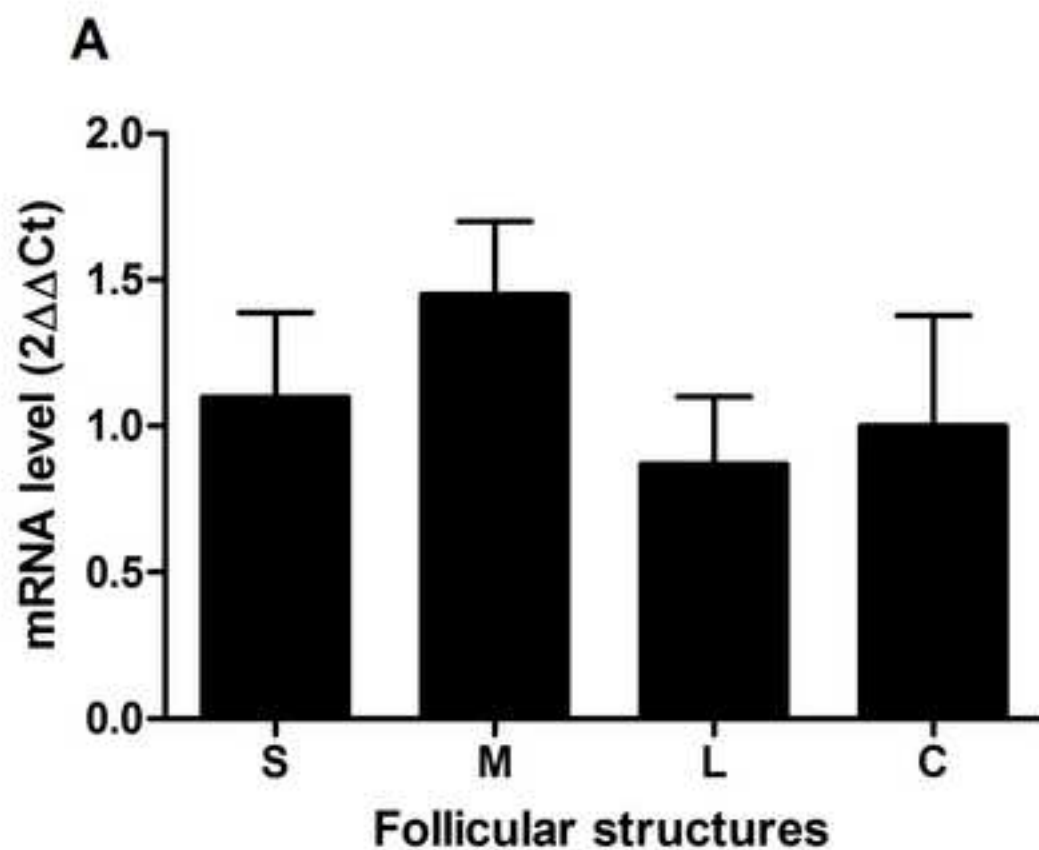
Table 2: Probe sequences (5'→3')

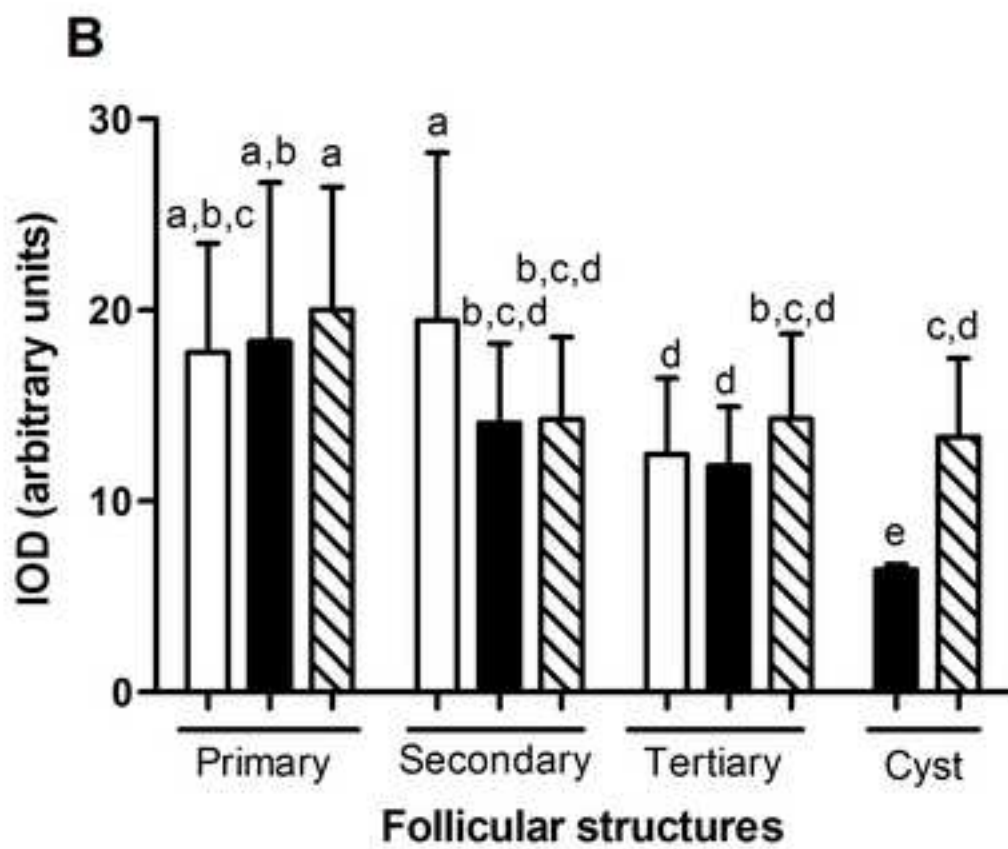
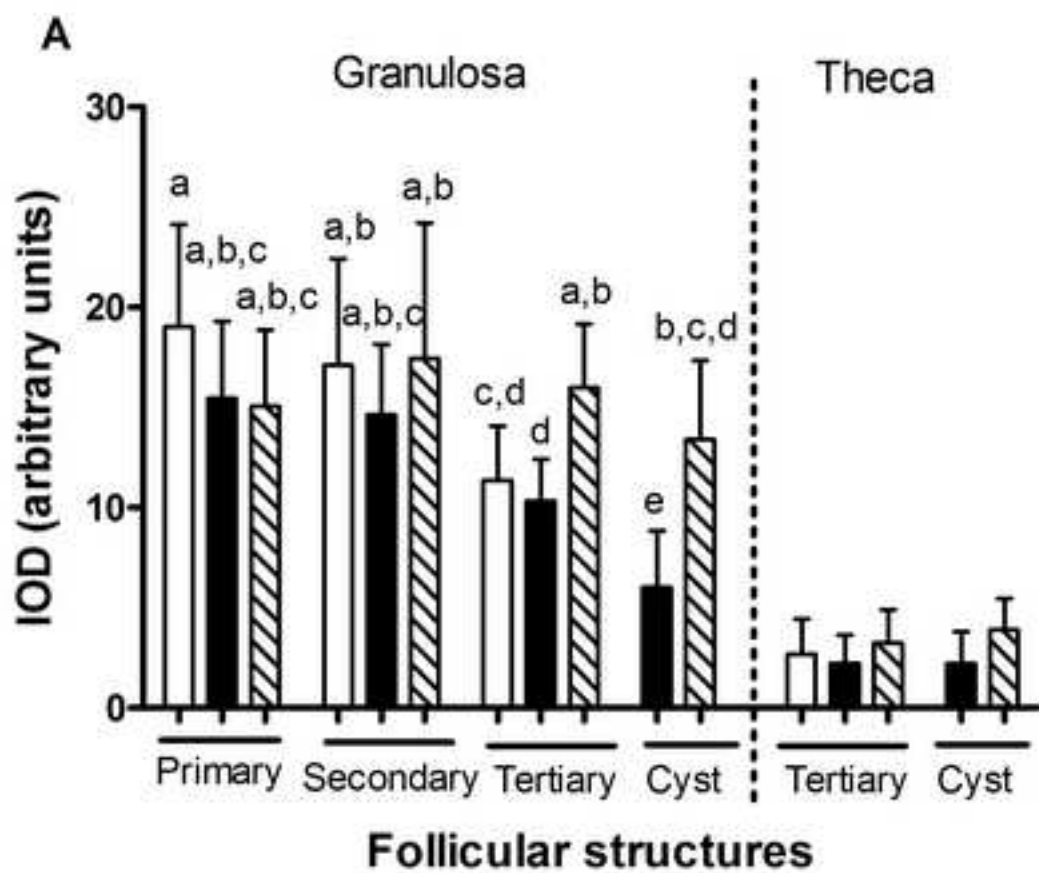
Probe	Sequence (5'→3')	Reference
IGF1	TCACATCCTCCTCGCATCTCTTCTATCTGGCCCTGTGCTTGCTCG	(Llewellyn et al., 2008)
IGFBP4	AAGACGGGAGTGAAGCTTCCGGGGGGCCTGGAGCCGAAGGG GGAG	901–945 of bovine IGFBP4 mRNA (Moser et al. 1992)
IGFBP5	CTACTCGCCCAAGATCTTCCGGCCCAAGCACACCCGCATCTCCG A	291–335 of bovine IGFBP5 gene (Moser et al. 1992)

Table 3: Antibodies, suppliers and dilutions used

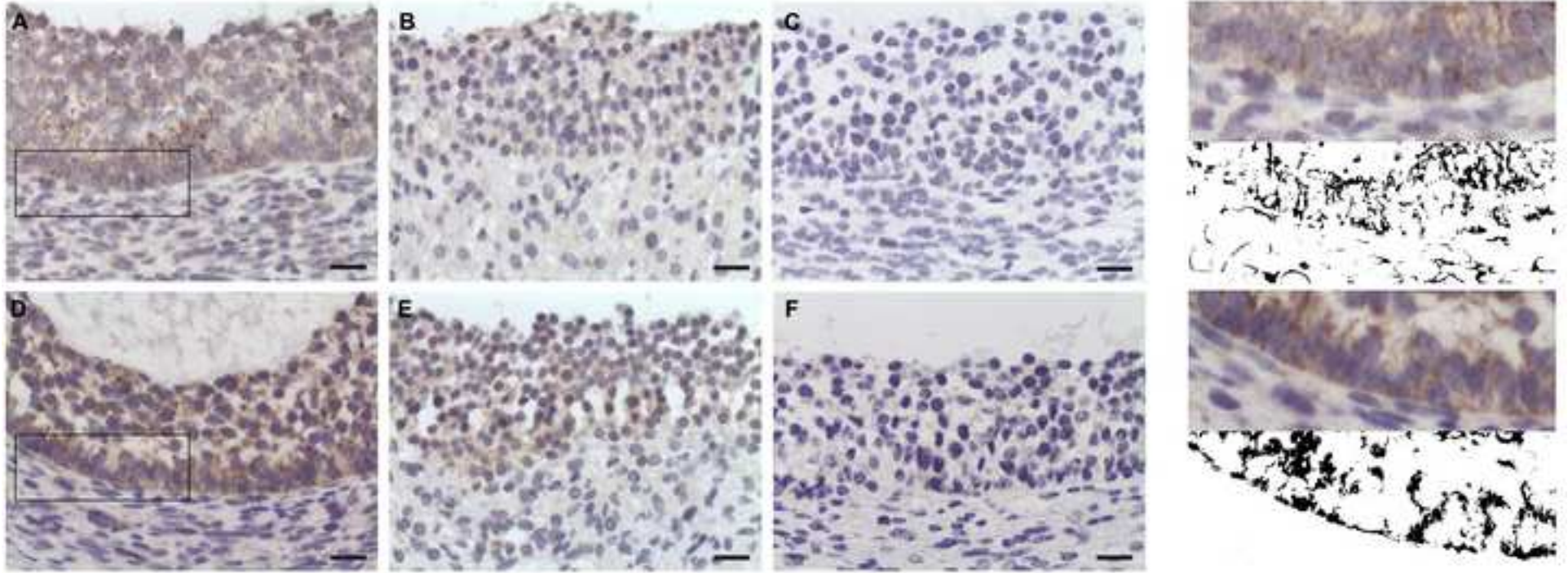
Antibodies	Type	Supplier	Dilution
<b>Primary antibodies</b>			
IGFBP1	Polyclonal	Novozymes GroPep Ltd, Australia	--
IGFBP4	Polyclonal	Novozymes GroPep Ltd, Australia	1:100
IGFBP5	Polyclonal	Novozymes GroPep Ltd, Australia	1:75
IGFBP6	Polyclonal	Novozymes GroPep Ltd, Australia	1:200
<b>Secondary antibody</b>			
Anti-rabbit IgG	Polyclonal	Zymed, CA, USA	1:200



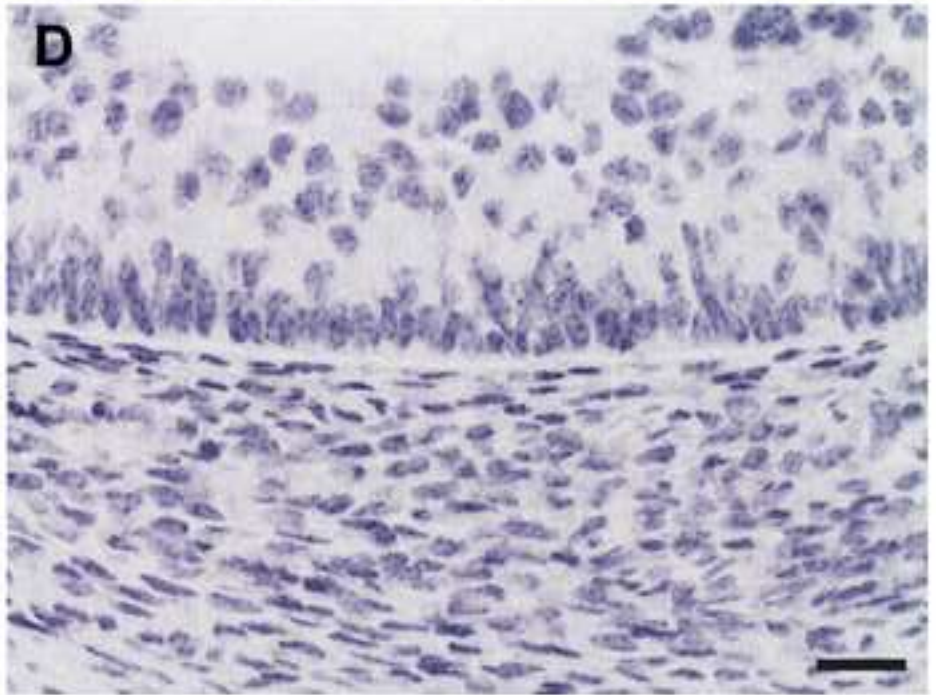
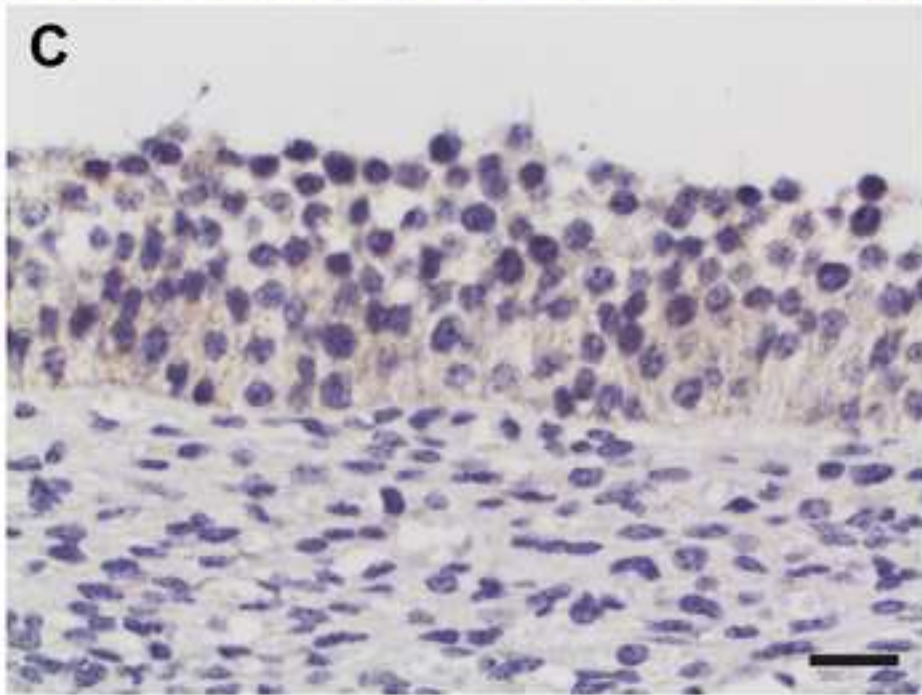
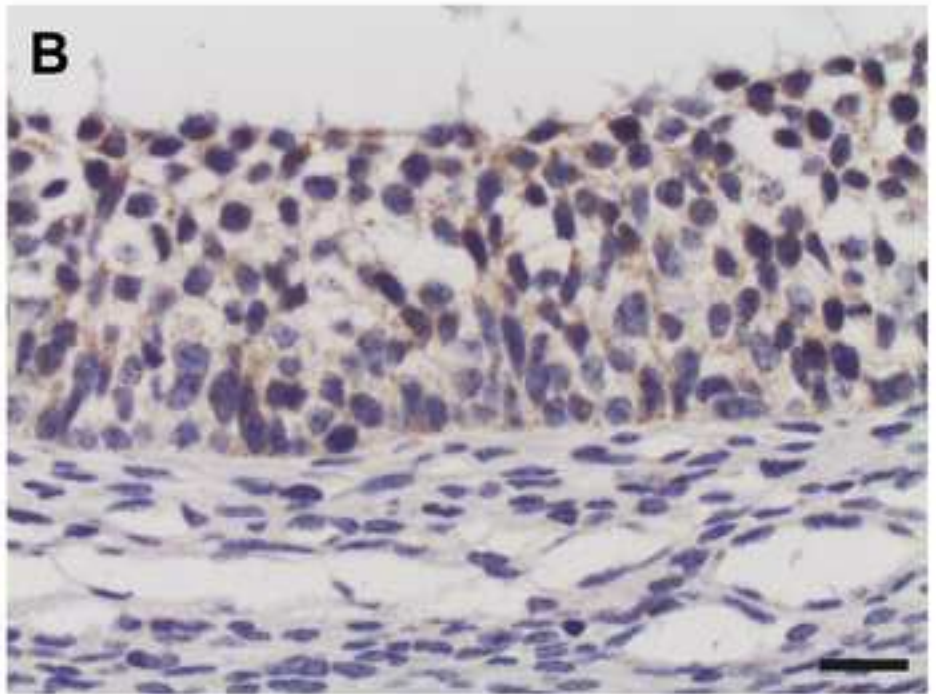
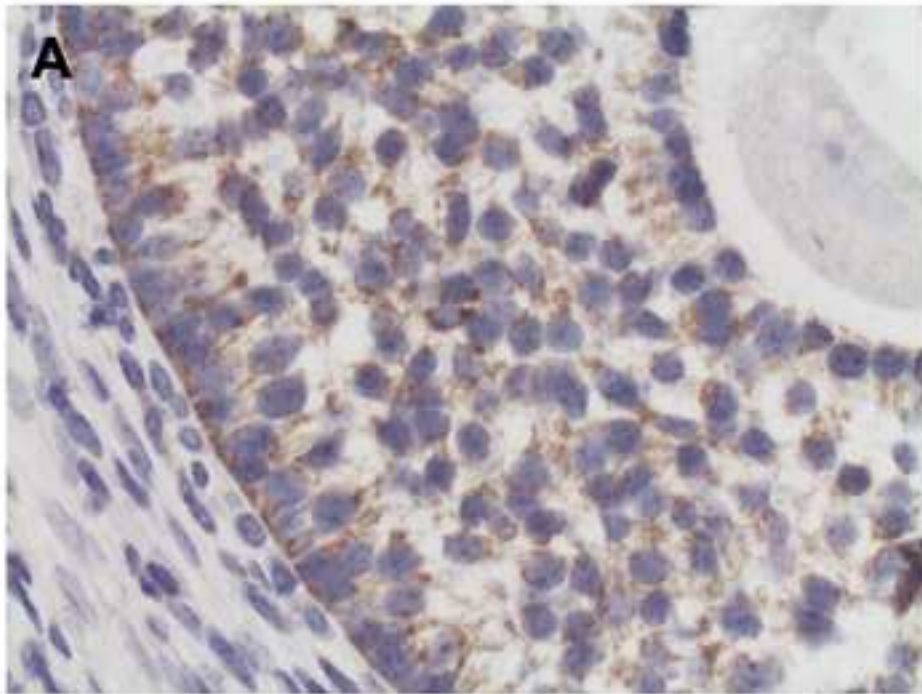




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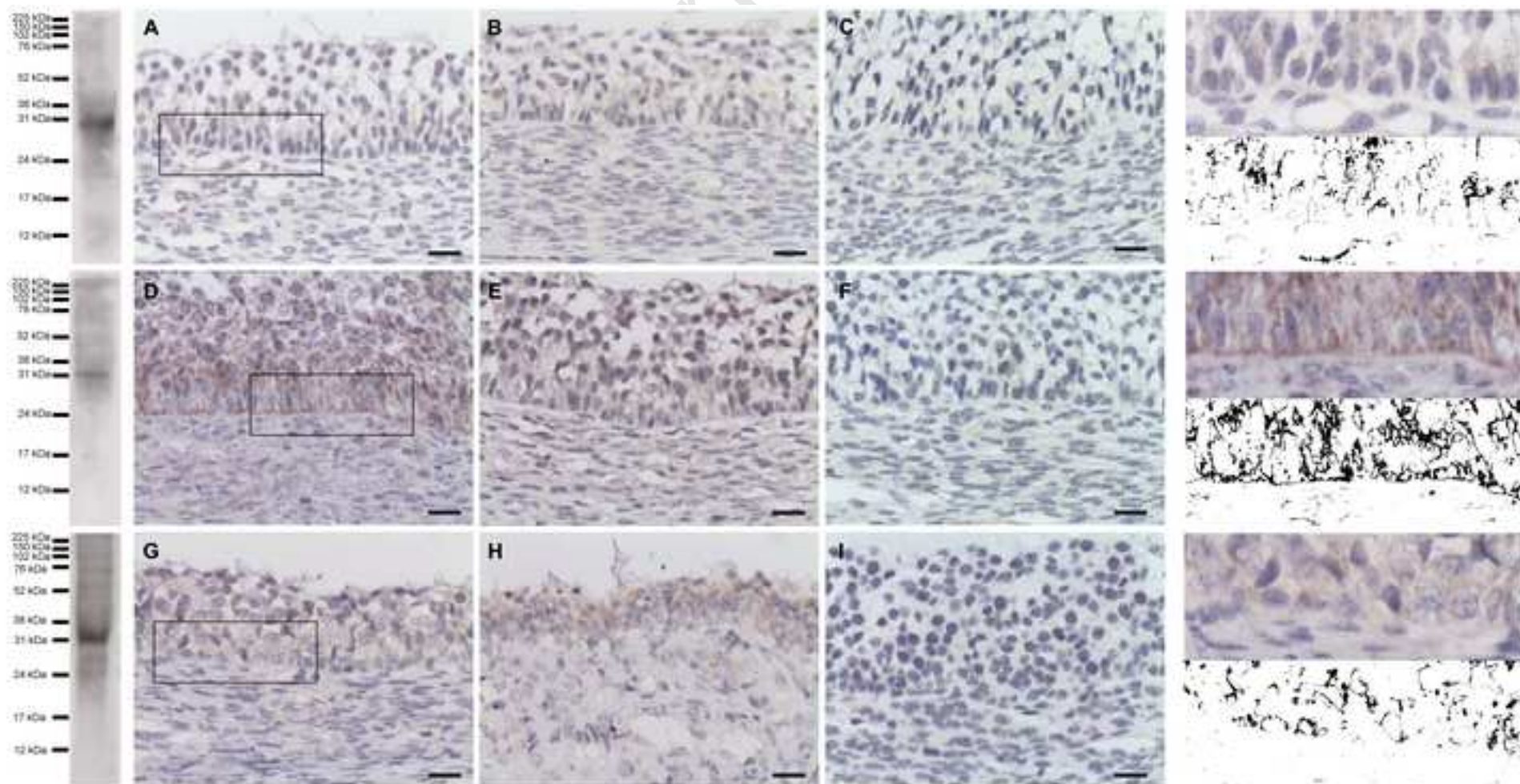


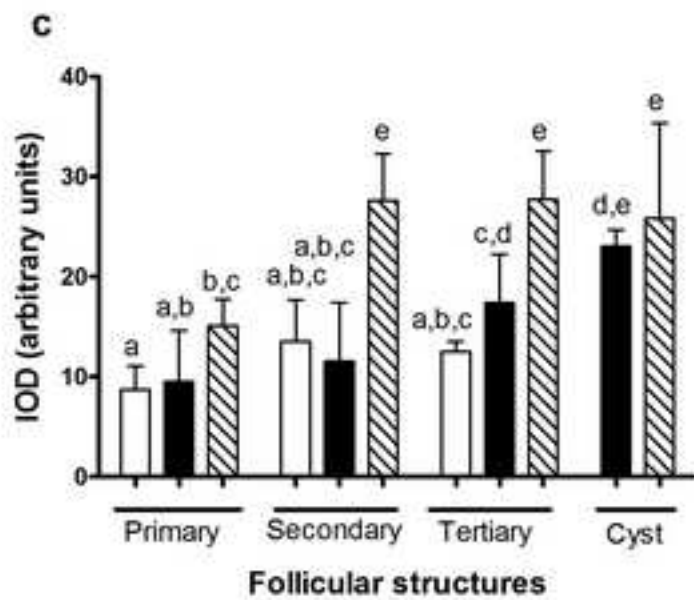
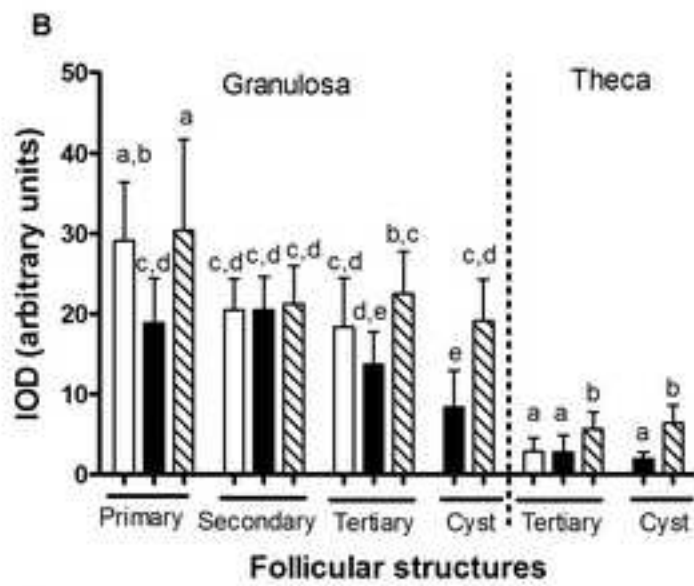
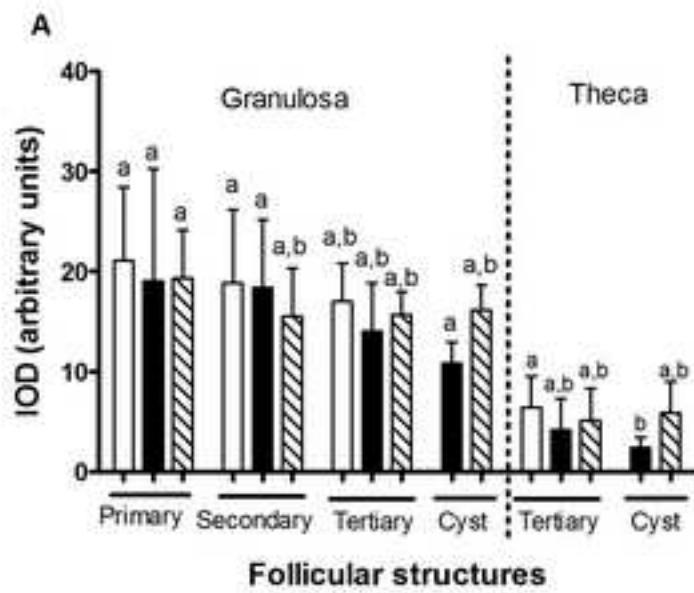


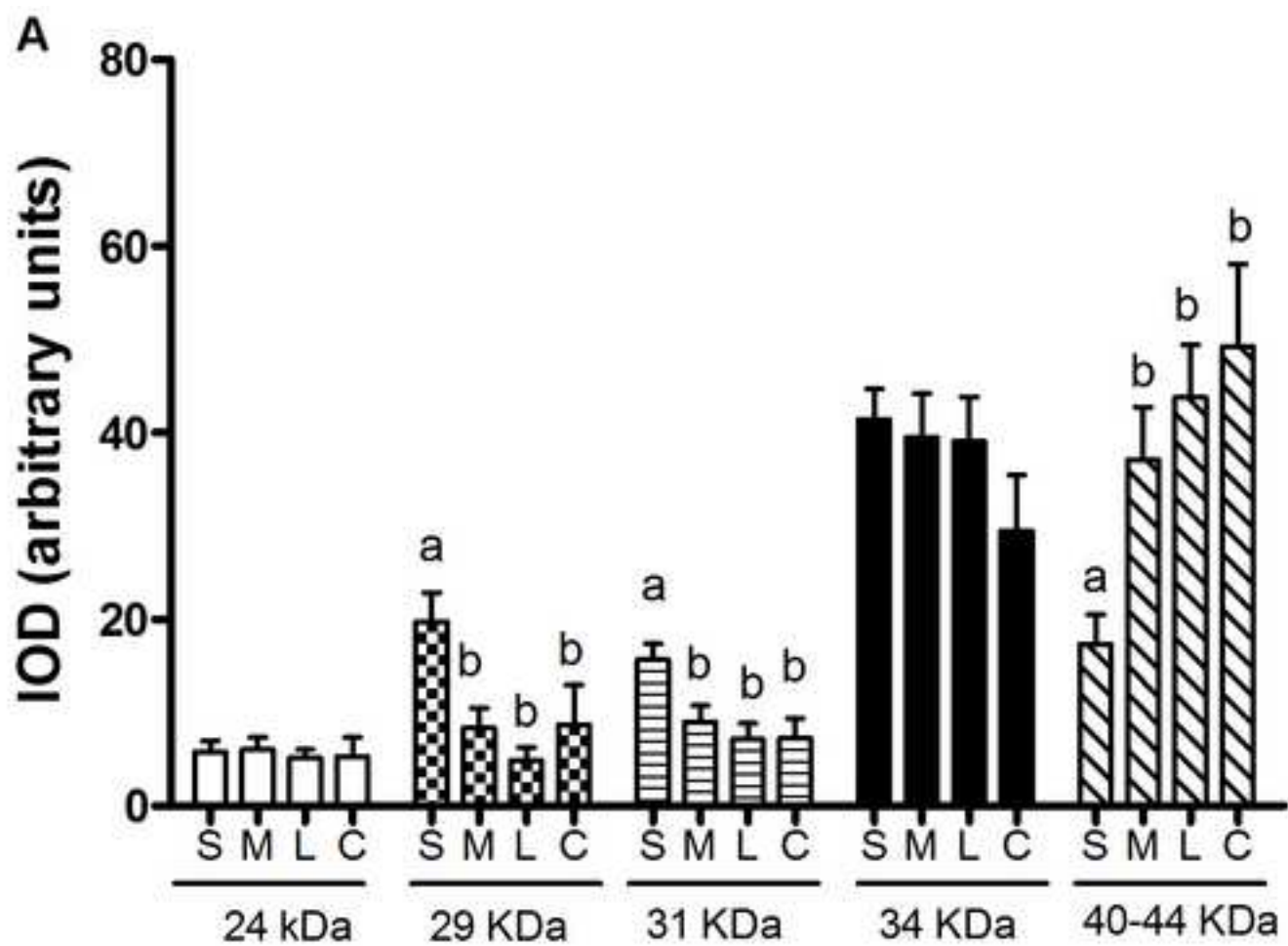




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